The NTPase/helicase activities of *Drosophila* maleless, an essential factor in dosage compensation

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***Drosophila* maleless (mle) is required for X chromosome dosage compensation and is essential for male viability. Maleless protein (MLE) is highly homologous to human RNA helicase A and the bovine counterpart of RNA helicase A, nuclear helicase II. In this report, we demonstrate that MLE protein, overexpressed and purified from Sf9 cells infected with recombinant baculovirus, possesses RNA/DNA helicase, adenosine triphosphatase (ATPase) and single-stranded (ss) RNA/ssDNA binding activities, properties identical to RNA helicase A. Using site-directed mutagenesis, we created a mutant of MLE (mle-GET) that contains a glutamic acid in place of lysine in the conserved ATP binding site A. In vitro biochemical analysis showed that this mutation abolished both NTPase and helicase activities of MLE but affected the ability of MLE to bind to ssRNA, ssDNA and guanosine triphosphate (GTP) less severely. In vivo, mle-GET protein could still localize to the male X chromosome coincidentally with the male-specific lethal-1 protein, MSL-1, but failed to complement mle¹ mutant males. These results indicate that the NTPase/helicase activities are essential functions of MLE for dosage compensation, perhaps utilized for chromatin remodeling of X-linked genes.**

Keywords: dosage compensation/helicase/MLE/NTPase/X chromosome

Introduction

Dosage compensation is the process by which the expression of X-linked genes is equalized between males and females. In *Drosophila*, dosage compensation is achieved by elevating the transcription of most genes on the single male X chromosome to a level equivalent to that of genes on the two female X chromosomes (Mukherjee and Beerman, 1965; Baker *et al.*, 1994). In contrast, in mammals, dosage compensation is achieved by transcriptional inactivation of genes on one of the two female X chromosomes (Ballabio and Willard, 1992; Penny *et al.*, 1996) and in nematodes, each X in the XX hermaphrodite is active but repressed relative to a single male X (Meyer and Casson, 1986; Kelley and Kuroda, 1995).

The initial step in establishing dosage compensation in *Drosophila* occurs early in development in response to

the ratio of X chromosomes to the haploid set of autosomes (the X:A ratio) (Maroni and Plaut, 1973). This process controls the expression of the developmental regulator, *Sex lethal* (*Sxl*). When the X:A ratio is 1.0, *Sxl* expression is directed by female-specific transcriptional activation of an early embryonic promoter; later, a positive autoregulatory loop is formed that maintains *Sxl* expression in females (Bell *et al.*, 1991; Keyes *et al.*, 1992). Active *Sxl* results in the female-specific splicing of late *Sxl* transcripts generated from a constitutive promoter (Bell *et al.*, 1991).

When the ratio of X:A is 0.5, *Sxl* protein is not expressed and male-specific regulators act to maintain equivalent levels of transcripts of most X-linked genes in males relative to females (Cline, 1978; Gorman *et al.*, 1993). In *Drosophila*, there are four such male-specific regulatory genes, named for their mutant phenotypes: *male-specific lethal-1* (*mle-1*), *male-specific lethal-2* (*mle-2*), *maleless* (*mle*) and *maleless on the third* (*mle-3*, also known as *mle-3*) (for references, see Baker *et al.*, 1994). *Sxl* is proposed to negatively regulate dosage compensation in females through the post-transcriptional regulation of the *mle-2* transcript, thereby repressing MSL-2 protein synthesis (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995). As a result, MLE, MSL-1 and MSL-3 proteins are present in both sexes (Gorman *et al.*, 1995), whereas MSL-2 protein is only found in males (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995).

These four positive regulators of dosage compensation (collectively called the MSL proteins) bind to hundreds of specific sites along the X chromosome only in males (Kuroda *et al.*, 1991; Palmer *et al.*, 1993; Hilfiker *et al.*, 1994; Bashaw and Baker, 1995; Gorman *et al.*, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995). Histone H4 acetylated at lysine 16 is also found localized at numerous sites along the length of the male X chromosome, largely coincident with the MSL proteins (Turner *et al.*, 1992; Bone *et al.*, 1994). The concentration of this modified histone on the male X chromosome requires the wild-type products of the *mle/mle* genes. Thus, it has been postulated that dosage compensation in *Drosophila* involves complex formation of the four MSL proteins on the male X chromosome that alters the chromatin structure, resulting in increased transcription (Turner *et al.*, 1992; Gorman *et al.*, 1993; Bone *et al.*, 1994).

As the biochemical mechanism of dosage compensation has not been defined, it is of considerable interest to characterize potential activities of the MSL proteins that may contribute to *in vivo* function. MLE belongs to the DEXH subfamily of nucleoside triphosphatase (NTPase) and/or helicase proteins (Kuroda *et al.*, 1991). It displays more similarity to RNA- rather than DNA-dependent NTPases or helicases, and has an additional motif characteristic of double-stranded (ds) RNA binding proteins

Fig. 1. Sedimentational analysis of MLE. An aliquot (53 μ g) of the pooled hydroxylapatite fractions enriched in recombinant MLE was loaded onto a 20–40% glycerol gradient and centrifuged for 36 h at 45 000 r.p.m. in a Beckman SW50.1 rotor at 4°C. Subsequently, 30 fractions were collected from the bottom of the tube. (A) The distribution of protein in the glycerol gradient. Aliquots (12 μ l) of each fraction were analyzed by electrophoresis on a 7.5% discontinuous SDS–polyacrylamide gel. Proteins were visualized by Coomassie blue staining. Lane M, molecular weight markers; lane L, 1 μ g of the hydroxylapatite fraction. All other lanes represent the fraction number from the glycerol gradient. (B) An immunoblot analysis carried out with polyclonal anti-MLE 25 antibodies and aliquots (2 μ l) of the indicated glycerol fractions. The distribution of helicase activities through the glycerol gradient is shown in (C) and (D). Unwinding reactions were performed with 50 fmol of partial duplex RNA or DNA substrate in the presence of the indicated glycerol fraction (0.1 μ l) or 10 ng of MLE (lanes 3 and 4). After incubation for 30 min at 37°C, the substrate and displaced single-stranded products were analyzed by electrophoresis on an 8% polyacrylamide gel (30:1) containing 5% glycerol and 0.5 $\times$ TBE. (C) An autoradiogram representing the distribution of DNA helicase activity in the glycerol gradient. Lane 1, substrate alone; lane 2, boiled substrate; lane 3, 10 ng of MLE without ATP; lanes 4–17, with ATP; lane 4, 10 ng of MLE; lanes 5–17, odd numbers of glycerol fractions 3–27. (D) The quantitation of results described in (C) and also of RNA helicase reactions performed with aliquots (0.1 μ l) of the indicated glycerol fractions as described above.

(Gibson and Thompson, 1994). Furthermore, the association of MLE with the X chromosome is RNase sensitive (Richter *et al.*, 1996). However, previous analyses of *mle* mutants were inconclusive regarding the importance of helicase motifs *in vivo* (Richter *et al.*, 1996) and no biochemical characterization of MLE protein has been documented.

In this report we demonstrate that MLE has NTPase and both RNA and DNA helicase activities. Furthermore, we show that loss of the NTPase and helicase activities of MLE results in male lethality in *Drosophila* without eliminating the localization of the MLE–MSL complex to the male X chromosome. These results indicate that the NTPase/helicase activities of MLE are essential for dosage compensation.

Results

Both DNA and RNA helicase activities are associated with MLE

Drosophila maleless shares extensive homology with human RNA helicase A (Lee and Hurwitz, 1993) and its bovine homolog nuclear DNA helicase II (Zhang *et al.*, 1995), two enzymes that possess helicase and NTPase activities (Lee and Hurwitz, 1992; Zhang and Grosse, 1994). For this reason, we assayed recombinant histidine-tagged MLE protein for these activities *in vitro*. MLE protein was isolated from Sf9 cells infected with the recombinant baculovirus. After adsorption to and elution from a nickel column, followed by chromatographic purification through hydroxylapatite (see Materials and

Fig. 2. Substrate specificity of MLE helicase activity. The structures of the different substrates used are shown at the top of each panel. All substrates were constructed using mRNA transcripts synthesized *in vitro* and synthetic DNAs of the same size and sequence as described previously (Lee and Hurwitz, 1992). Unwinding reactions (20 μ l) containing 50 fmol of the indicated substrate were incubated in the presence of increasing amounts of MLE. Lane 1, substrate alone; lane 2, boiled substrate; lane 3, 5 ng of MLE without ATP; lanes 4–6, 1.25, 2.5, 5 ng of MLE respectively, in the presence of ATP.

methods), the isolated recombinant protein was subjected to glycerol gradient sedimentation (Figure 1). MLE, which sedimented between bovine serum albumin (BSA) (4.3S) (fraction 12) and aldolase (7.4S) (fraction 19), peaked at fraction 16 with an estimated sedimentation coefficient of 6.2S (Figure 1A). Immunoblot analysis with polyclonal antibodies specific for MLE established that the major 140 kDa protein in the purified hydroxylapatite and peak glycerol fractions was full-length MLE (Figure 1B). Aliquots (0.1 μ l) of each fraction were tested for both RNA and DNA helicase activities. Both activities, which sedimented coincidentally, were detected in fractions enriched in MLE (Figure 1C and D), demonstrating that these activities are intrinsically associated with MLE.

In order to determine the substrate specificity of MLE, four different substrates, containing identical ribonucleotide or deoxyribonucleotide sequences as shown in Figure 2 (RNA:RNA, RNA:DNA, DNA:RNA and DNA:DNA hybrids), were constructed and tested in the unwinding reaction with varying amounts of MLE (2.5–10 ng). MLE displaced substrates containing ssRNA regions, i.e. RNA:RNA and DNA:RNA hybrids, 2.5-fold more efficiently than substrates containing ssDNA regions (Figure 2).

The binding of MLE to ssRNA and ssDNA was examined (Figure 3). For this purpose, the longer strand of each duplex substrate described in Figure 2 (the 98mer ssRNA or ssDNA) was tested using a gel mobility shift assay in the presence of increasing levels of MLE (5–20 ng) (Figure 3). In keeping with the higher helicase activity observed with duplex substrates containing ssRNA, complexes with MLE were formed more efficiently (3- to 4-fold) with ssRNA than with ssDNA.

Characteristics of helicase activities associated with MLE

The experiments described above demonstrated that MLE possesses RNA and DNA helicase activities. Previously, we reported that RNA helicase A, the human homolog of MLE, contained no detectable DNA helicase activity (Lee and Hurwitz, 1991). In contrast, Grosse's laboratory detected both RNA and DNA helicase activities with

Fig. 3. The binding of MLE to ssRNA and ssDNA. The binding of MLE to ssRNA or ssDNA was measured in reaction mixtures (20 μ l) containing 50 fmol of substrate and varying amounts of MLE, as described previously (Lee and Hurwitz, 1992). Complexes formed between MLE and ssRNA (left panel) or ssDNA (right panel) were quantitated following autoradiography after electrophoretic separation. The amounts of RNA or DNA complexed with MLE (bracketed regions) are presented at the bottom of each lane. Lane 1: substrate alone; lanes 2–4: 5, 10, 20 ng of MLE respectively.

bovine nuclear helicase II, which is >90% identical to HeLa RNA helicase A (Zhang *et al.*, 1995). In our earlier experiments with RNA helicase A, KCl or NaCl (50 mM) was added to unwinding reaction mixtures, which optimized the unwinding of partial duplex RNA substrates by RNA helicase A. Zhang and Grosse (1994) reported that DNA helicase activity of bovine nuclear helicase II was more salt sensitive than the RNA helicase activity. Indeed, as described by Zhang and Grosse (1994), RNA helicase A was found to contain DNA helicase activity that is more salt sensitive than RNA helicase activity (data not shown). In light of these findings, the influence of salt on RNA and DNA helicase activities of MLE was examined. As shown in Figure 4A, DNA helicase activity was markedly inhibited by 0.1 M NaCl, whereas RNA helicase activity was hardly affected by this salt concentration. At higher levels, however, both activities were inhibited.

Fig. 4. Properties of MLE helicase activity. (A) The influence of salt on the helicase activities of MLE. All reactions were carried out with 10 ng of MLE and 50 fmol of partial duplex RNA or DNA substrates in the presence of the indicated concentrations of NaCl. (B) The influence of various NTPs on MLE helicase activity. The unwinding reactions (20 μ l) were carried out with 10 ng of MLE and 50 fmol of partial duplex RNA or DNA substrates in the presence of 1 mM of the indicated NTP. In the presence of 1 mM ATP, 37 fmol of ssRNA and 23 fmol of ssDNA were formed; there was no significant change in the efficiency of the unwinding reaction in the presence of other NTPs.

The helicase activity of MLE required adenosine triphosphate (ATP) and in its absence, partial duplex substrates were not displaced (Figure 2, lanes 3 and 4). In addition to ATP, all other seven common nucleoside triphosphates (NTPs) supported helicase activity (Figure 4B). The K_m for ATP in the helicase reaction was 10 μ M and similar values were obtained for the other NTPs (data not shown), indicating that MLE utilizes all NTPs without preference.

The partial duplex substrates described in Figure 2 contained single-stranded regions at both 3'- and 5'-ends. The directionality of a helicase is defined by the strand to which the enzyme binds and translocates. To determine the directionality of MLE helicase activity, three different substrates were prepared. The upper longer DNA of each substrate was the same but the lower shorter complementary DNA differed in each substrate. The duplex substrates B and C contained a single-stranded region exclusively at either the 3'- or 5'-end (Figure 5). The nucleotide sequence

of substrate D which contained no single-stranded region was identical to the duplex region present in substrate B. These three substrates were examined in the unwinding reaction in the presence of various levels of MLE (2.5–10 ng). As shown in Figure 5, the 3'-tailed substrate was efficiently displaced whereas unwinding was not observed with the 5'-tailed substrate (Figure 5B and C). As expected, the unwinding reaction mediated by MLE was completely dependent on the presence of a single-stranded region; substrate D, devoid of any single-stranded region, was not utilized by MLE (Figure 5D). The same 3' to 5' directionality was observed with duplex RNA substrates (data not shown), indicating that MLE recognizes and binds both ssRNA and ssDNA and translocates in the 3' to 5' direction. These properties are identical to those observed with RNA helicase A (Lee and Hurwitz, 1992).

Site-directed mutagenesis of ATP binding motif A of MLE

The results described above demonstrated that MLE possesses RNA/DNA binding activity, NTPase activity, and DNA/RNA helicase activities. Next we determined whether these activities are essential for the role MLE plays in dosage compensation in *Drosophila*. If NTPase and helicase were essential functions of MLE, mutations affecting these activities should also affect dosage compensation. In earlier studies, the most highly conserved region of the ATPase site A within the helicase domain GKT, was changed to GNT. This mutation reduced male viability ~50% (Richter *et al.*, 1996). Reasoning that a lysine to asparagine change in the ATP binding motif GKT may have been inadequate to completely eliminate the function of MLE, a more substantial change (lysine to glutamic acid) was introduced into MLE, which reversed the ionic charge from positive to negative in the ATPase site A.

In vitro analysis of the effect of the GET mutation in MLE

The six-histidine-tagged mle-GET, possessing the lysine to glutamic acid mutation in the GKT conserved helicase motif, was expressed and purified from Sf9 cells infected with the recombinant virus. The expression level of mle-GET was low, and only 34 μ g of mle-GET was isolated from 0.5 l of Sf9 cells after hydroxylapatite chromatography following Ni-affinity adsorption and elution (see Materials and methods). Hydroxylapatite fractions, highly enriched in mle-GET, were pooled (Figure 6, fractions 13–19) and used to characterize the biochemical properties of mle-GET. As shown in Figure 7, mle-GET contained no significant RNA-dependent NTPase activity or helicase activity with the DNA or RNA substrates. Since the unwinding reaction requires NTP hydrolysis for translocation and disruption of the duplex, the lack of helicase activity of mle-GET is most likely due to its inability to hydrolyze NTP.

Since the binding of MLE to single-stranded polynucleotides occurs in the absence of NTPs, we expected that the GET mutation would not affect the NTP-independent activities of MLE. As expected, the mle-GET bound both ssRNA (Figure 8A, lanes 5–7) and ssDNA (data not shown), though less efficiently (~2.5-fold) than wild-type MLE (Figure 8A, lanes 2–4). In the presence of ATP, the

Fig. 5. Directionality of MLE helicase activity. In addition to the standard substrate (A), three different partial duplex substrates (B–D) were tested in the unwinding reaction. The structure of each substrate is shown at the top of each panel. Lane 1, substrate alone; lane 2: boiled substrate; lane 3, 10 ng of MLE without ATP; lanes 4–6 contained ATP and 2.5, 5, 10 ng of MLE respectively.

Fig. 6. Purification of mle-GET by hydroxylapatite column chromatography. The pooled Ni-fraction (4 ml, 110 μ g of protein), enriched in six-histidine tagged mle-GET, was directly loaded onto the hydroxylapatite column (0.5 ml) and bound proteins were eluted with a linear gradient (20–300 mM) of sodium phosphate, pH 6.0, containing 0.25 M NaCl, 2 mM DTT, 0.05% NP-40 and 10% glycerol. A total of 40 fractions (each 0.25 ml) was collected and aliquots (10 μ l) were electrophoresed on a discontinuous 7.5% SDS–polyacrylamide gel. Fractions (13–19), enriched in mle-GET, were pooled and used in the present study following dialysis against 2 l of buffer containing 20 mM HEPES–NaOH, pH 7.4, 0.25 M NaCl, 0.1 mM EDTA, 0.5 mM PMSF, 2 mM DTT and 12.5% glycerol. Lane M, molecular weight marker; lane L, 0.3 μ g of the pooled Ni-fraction; lane F, the flow-through fraction; lanes 1–31, odd numbered hydroxylapatite fractions.

complexes of MLE and RNA (and also DNA) displayed subtle changes in their migration and in the relative amount of each complex formed (compare Figure 8A, lanes 4 and 11). The fast-migrating complex decreased with the concomitant increase in the slower-migrating complexes. A similar result was obtained with complexes formed with mle-GET (compare Figure 8A, lanes 7 and 14). This result raised the possibility that although NTP hydrolysis activity was absent, mle-GET could still bind NTPs.

UV-crosslinking (Pause *et al.*, 1993) was used to measure the binding of nucleotides to MLE. Under the conditions used (Figure 8B), GTP was crosslinked to MLE more efficiently than ATP, and the efficiency of GTP crosslinking to MLE was maximal at a nucleotide

concentration of 10 μ M. The addition of RNA or DNA hardly affected the UV-crosslinking of GTP to MLE (data not shown). The binding of GTP to MLE and mle-GET was examined in the presence of [α - 32 P]GTP. Increasing levels of MLE (20–80 ng) resulted in an increased binding of GTP (Figure 8B, lanes 2–4). It was estimated that 20 fmol and 4.7 fmol of GTP were crosslinked to 80 ng (570 fmol) of MLE and mle-GET, respectively (lanes 4 and 7). The results presented in Figure 8B demonstrated that mle-GET bound GTP with an efficiency ~5- to 10-fold lower than wild-type MLE.

In vivo analysis of the GET mutation

A genomic mle-GET P-element construct was injected into embryos, and 13 independent transgenic lines were

Fig. 7. The influence of the GET mutation on the NTPase/helicase activities of MLE. **(A)** The ATPase activity of MLE and mle-GET was examined. Reaction mixtures (15 μ l) containing 20 mM HEPES–NaOH, pH 7.4, 2 mM DTT, 3 mM $MgCl_2$, 0.1 mM [γ - ^{32}P]ATP (4400 c.p.m./pmol) and 0.2 mg/ml BSA, were incubated for 30 min at either 25°C or 37°C in the presence of increasing levels of each protein. Aliquots (1 μ l) of each reaction were analyzed on PEI plates and the extent of ATP hydrolysis was quantitated, as described previously (Lee and Hurwitz, 1992). **(B)** MLE and mle-GET were examined in unwinding reactions containing 50 fmol of either partial duplex RNA (left panel) or partial duplex DNA substrate (right panel). Lane 1, substrate alone; lane 2, boiled substrate; lanes 3 and 7, 10 ng of MLE and mle-GET respectively, without ATP; lanes 4–6 and 8–10 contained ATP and 2.5, 5, 10 ng of the indicated protein. In the presence of 10 ng of MLE, 39 fmol and 27 fmol of ssRNA and ssDNA were formed respectively. No substrate displacement was observed at any level of mle-GET protein added.

established. Three independent insertions of the transgene were examined by Western blot analysis, and all three lines expressed a full-length transgenic MLE protein (Figure 9 and data not shown). The three insertion lines were tested for the ability of the *mle*-GET transgene to rescue *mle*¹ males. Whereas a wild-type *mle* transgene fully complements the *mle*¹ mutant phenotype (Richter, 1994; Richter *et al.*, 1996), the percentage of *mle*¹ males rescued by the *mle*-GET transgene was very low. One line rescued at 1.3% ($n = 456$), the second line rescued at 0.7% ($n = 390$) and the third line did not rescue at all ($n = 223$). The majority of the *mle*¹ males with the *mle*-GET transgene died during mid to late larval stages, as do *mle*¹ males lacking the transgene. The *mle*¹ males that survived to adulthood were sterile, held out their wings and had disorganized abdominal bristles. Each of these phenotypes has been observed previously in rare surviving *mle* males carrying severe loss of function alleles (M.Kuroda, unpublished data). The low level of rescue

suggests that the *mle*-GET mutation disrupts MLE function. MLE is thought to associate with the X chromosome in a complex with the other MSLs, and to act within that complex to facilitate dosage compensation.

One copy of a wild-type *mle* transgene in *mle*¹ mutant males is sufficient to produce co-localized MLE and MSL-1 immunostaining patterns that are indistinguishable from wild-type males (Richter, 1994). To determine if the *mle*-GET protein was able to assemble in an MSL complex on the X, the localization of MLE and MSL-1 was determined on polytene chromosomes obtained from *w*; *mle*¹; [*w*⁺; *mle*-GET] male third instar larvae. The sole source of MLE in these larvae is from the *mle*-GET transgene, as *mle*¹ mutants have no detectable MLE by Western blot or on polytene chromosomes (Richter *et al.*, 1996). The staining results indicated that the *mle*-GET protein was able to bind the X chromosome (Figure 10A). The number of sites where the *mle*-GET protein associated with the X chromosome was reduced from the number seen in wild-type males, and the number varied from one male to the next. The MSL-1 pattern was also reduced, as is seen in *mle* mutants (Palmer *et al.*, 1994). The *mle*-GET protein co-localized with all MSL-1 sites and also stained additional sites on the X and the autosomes (Figure 10B). We cannot determine whether the reduced number of the X chromosome sites and the increased staining of autosomal sites has functional significance, or reflects an indirect effect of the altered physiology of dying larvae. However, our results demonstrate that the *mle*-GET mutation does not dramatically alter MLE protein conformation, as *mle*-GET can still bind to the X chromosome coincident with MSL-1. That the *mle*-GET protein was able to form complexes on the X chromosome but unable to support a wild-type MSL pattern on the X or to rescue *mle* males, suggests that the NTPase/helicase activities are essential for the function of MLE in dosage compensation.

Discussion

MLE and RNA helicase A are biochemically equivalent

We have shown that both NTPase and helicase activities are intrinsically associated with MLE. Partial duplex substrates tested in the unwinding reaction, including those containing RNA:RNA, DNA:DNA and RNA:DNA duplexes, were efficiently displaced by MLE in an NTP-dependent manner. The requirement of a single-stranded region for the helicase activity of MLE is in keeping with its ability to bind ssRNA and ssDNA and its inability to bind the duplex RNA or DNA tested. MLE formed stable complexes with ssRNA and ssDNA in the absence of NTP. However, as observed with RNA helicase A (Lee and Hurwitz, 1991), MLE rapidly dissociated from these complexes upon addition of ATP and an unlabeled single-stranded polynucleotide competitor (data not shown). These results suggest that single-stranded regions present in partial duplex substrates are essential for the unwinding reaction mediated by MLE. We suggest that MLE recognizes and binds these regions and that NTP hydrolysis is required for the translocation of MLE in a 3' to 5' direction along the single-stranded region and through duplex regions in which hydrogen bonds must be disrupted during unwinding.

Fig. 8. The influence of the GET mutation on the binding of MLE to ssRNA and GTP. **(A)** The binding of MLE and mle-GET to ssRNA was examined in reactions containing 50 fmol of substrate in the absence (lanes 1–7) or presence (lanes 8–14) of ATP. After incubation for 30 min at 37°C, aliquots (10 μ l) were loaded onto a 4.5% polyacrylamide gel (60:1) containing 5% glycerol in 0.5 $\times$ TBE and electrophoresed at 15 mA for 1.5 h. Lanes 1 and 8: substrate alone; lanes 2–4 and 9–11: 5, 10, 20 ng of MLE respectively; lanes 5–7 and 12–14, 5, 10, 20 ng of mle-GET respectively. **(B)** The binding of MLE and mle-GET to GTP was examined in reactions (20 μ l) containing [α - 32 P]GTP (10 μ M, 5500 c.p.m./pmol) and increasing levels of protein (20–80 ng); reactions were incubated on ice for 10 min and then irradiated with UV-light (254 nm) for 10 min on ice. Formation of the GTP–protein complex was analyzed by SDS–PAGE and visualized by autoradiography. Lane 1: no protein; lanes 2 and 5: 20 ng of protein; lanes 3 and 6: 40 ng of protein; lanes 4 and 7: 80 ng of protein.

Each of the eight common NTPs efficiently supported MLE helicase activity. A Lineweaver–Burk plot of MLE helicase activity indicated a K_m of 10 μ M for ATP; similar results were obtained with other NTPs. The biochemical activities of MLE are almost identical to those of human RNA helicase A and bovine DNA helicase II (Lee and Hurwitz, 1991; Zhang and Grosse, 1994). These findings, together with their sequence similarity (49% identity and 89% homology across the entire sequence) (Lee and Hurwitz, 1993; Zhang *et al.*, 1995), suggest that these proteins perform their functions through the same biochemical mechanism.

Chromosomal binding and NTPase/helicase activities are separable functions of MLE

DNA and RNA unwinding reactions constitute key steps in various nucleic acid transactions. The DEAD/DEAH-box family of proteins is essential for these processes and has been identified in diverse organisms from *Escherichia coli* to humans (Gorbalenya *et al.*, 1989; Koonin, 1991; Wassarman and Steitz, 1991). As a member of this family, MLE has been of particular interest as a developmental regulator whose biological role has been relatively well defined. Our *in vitro* biochemical and *in vivo* complementation analyses indicate that the introduction of the GET mutation into the MLE ATP binding motif A abolishes NTPase/helicase activities and the ability to support dosage compensation. Transgenic male larvae harboring mle-GET as their sole source of MLE, produced the mutant protein but were inviable.

Although NTPase/helicase activities were eliminated, the mle-GET protein retained some affinity for GTP and bound ssRNA and ssDNA efficiently *in vitro*. In addition, the mle-GET protein could bind the X chromosome *in vivo*, suggesting that its chromosomal localization does not

Fig. 9. Western blot of MLE protein in flies carrying the mle-GET transgene. Lane 1, control nuclear extract from Schneider cells; lane 2, total protein from adult mle¹ escaper males carrying one copy of the mle-GET transgene; lane 3, total protein from adult mle¹ females carrying one copy of the mle-GET transgene. Anti-MLE antibody was used to detect the MLE protein.

strictly require the NTPase/helicase activities. However, mutant MLE and wild-type MSL-1 bound to fewer sites on the X in mle-GET mutants, and mle-GET protein was also detected at an increased number of autosomal sites, suggesting that formation or stability of the mutant mle-GET–MSL complex is less efficient than wild type. This might be due to some loss of interaction of MLE with the other MSL proteins that would result in diminished X chromosome specificity for MLE, concomitant with loss of the full pattern of MSL staining for the other complex members (Palmer *et al.*, 1994). Previous studies have

Fig. 10. Analysis of the ability of the *mle-GET* protein to bind to the male X chromosome. Indirect immunofluorescence was performed on polytene chromosomes from male salivary glands. Salivary glands were squashed from a wild-type male and a *w; mle¹:[mle-GET]/+* male. The chromosomes were stained with antibodies to MLE and MSL-1. The staining was visualized through the use of Texas Red and FITC conjugated secondary antibodies. The top row (**A**) shows the staining with the anti-MLE antibody. The bottom row (**B**) shows the double staining with anti-MLE and anti-MSL-1 antibodies. Where the red MLE staining overlaps with the green MSL-1 staining, a yellow color is observed.

indicated that MLE is absolutely dependent on MSL-1 and MSL-2 for its X chromosomal specificity (Gorman *et al.*, 1993). Furthermore, overexpressed MLE, or the C-terminal 353 amino acids of MLE (including glycine-rich heptad repeats but lacking the helicase domain) bind all chromosomes indiscriminately, probably through association with RNA (Richter *et al.*, 1996). Thus, NTPase/helicase activities of MLE are not essential for its chromosome association, but may play a role in its stable interaction with other dosage compensation regulators.

The role of MLE and MSLs in dosage compensation

MLE and the other MSL proteins mediate dosage compensation by increasing the rate of transcription of genes on the male X chromosome. The MSL proteins are required for specific histone H4 acetylation on the X

chromosome (Bone *et al.*, 1994) and recent studies have provided strong evidence that histone acetylation plays an important role in transcriptional activation (Lee *et al.*, 1993; Brownwell *et al.*, 1996; Wolffe *et al.*, 1996). Thus, it is likely that the MSL proteins will facilitate increased transcription through chromatin remodeling. The sequences of the MSL proteins contain several motifs potentially associated with transcriptional control. MSL-1 contains an acidic N-terminal domain (Palmer *et al.*, 1993). MSL-2 is a RING finger family member possessing a cysteine-rich zinc-binding motif found in a number of proteins involved in transcriptional regulation, DNA recombination and DNA repair (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995; Saurin *et al.*, 1996). MSL-3 contains two chromo domains (Koonin *et al.*, 1995), another motif associated with chromosomal proteins involved in transcriptional control. So far, MLE is the only

dosage compensation regulator with defined biochemical activities, and these suggest that it could have several roles in transcriptional regulation. Its DNA helicase activity may contribute to destabilizing chromatin structure, analogous to ATP-dependent nucleosomal disruption by the SWI/SNF and *Drosophila* NURF complexes (Peterson and Herskowitz, 1992; Yoshinaga *et al.*, 1992; Cote *et al.*, 1994; Tsukiyama and Wu, 1995; Tsukiyama *et al.*, 1995). Its RNA helicase activity could facilitate transcription by altering the structure of nascent RNA, a process that can stimulate reinitiation and/or elongation. Alternatively, MLE may interact with a hypothetical structural RNA component of the chromosome. That an interaction with RNA is important is demonstrated by the RNase sensitivity of MLE association with the polytene X chromosome (Richter *et al.*, 1996). Interestingly, while MLE is released following RNase treatment, the other MSL proteins remain associated with the chromosome, suggesting that both RNA and DNA-mediated recognition of the X chromosome are critical for dosage compensation.

Though dosage compensation in *Drosophila* is a highly specific process used to up-regulate the male X chromosome, a reverse phenomenon is used to down-regulate transcription of female X chromosomes in other species (Meyer and Casson, 1986; Ballabio and Willard, 1992; Penny *et al.*, 1996). In *Drosophila*, increased RNA synthesis is the chief function of dosage compensation whereas the inhibition of transcription by *Xist* RNA is the means by which inactivation of the mammalian female X chromosome occurs (Ballabio and Willard, 1992; Penny *et al.*, 1996). Both processes are likely to be dependent on the functional states of chromatin (Turner *et al.*, 1992; Jeppesen and Turner, 1993) and the action of specific transcriptional regulators. In the case of female X chromosome inactivation, regulators remain to be identified. However, in *Drosophila*, a number of these regulators have been identified but their precise function remains unclear. Our results indicate that the helicase activity of MLE is one important activity in this process. *In vivo*, it is evident that MLE is highly dependent on the other MSL proteins for X chromosome localization, and perhaps also for specificity of function. The availability of highly purified, enzymatically active MLE, as well as cloned MSL proteins, should provide important means for elucidating the biochemical properties of these proteins and their role in dosage compensation.

Materials and methods

Fly stocks

The *mle^l* allele (Fukunaga *et al.*, 1975) which produces no detectable MLE protein and is recessive lethal to males, was used for complementation studies. The characteristics of *yw* flies and the *CyO* balancer chromosome are documented in Lindsley and Zimm (1992).

Site-directed mutagenesis

Mutagenesis was used to alter the coding sequence within the ATPase site A of *mle*, substituting the conserved lysine residue with a glutamic acid (GKT to GET). For this purpose, a 5 kb fragment containing the ATPase region of *mle* was subcloned into pBluescript II KS⁺ (Stratagene), which was used as a template for PCR mutagenesis (Higuchi *et al.*, 1988). Two primer sets were designed to perform the mutagenesis. Primer set one consisted of a primer 5' to the ATP binding site A paired with a primer containing the mutation in the ATP binding site A. Primer set two contained the reverse complement of the mutant primer in set

one and a primer 3' of the ATP binding site A. The products from these two initial primer sets were pooled together in equal molar concentrations, denatured and allowed to reanneal to one another in the region of the mutant primers. The annealed mutant fragments were extended using *Taq* polymerase (Perkin-Elmer Cetus) to form a full-length dsDNA. The double-stranded mutant fragment was amplified by adding the 5' and 3' primers to the reaction. The 800 bp PCR product was digested with restriction enzyme *SalI* and substituted back into the pBluescript subclone. The PCR mutagenized fragment was sequenced to confirm the presence of the mutation and to ensure that no additional mutations had been introduced. The 5 kb subcloned fragment was then substituted into a previously described *mle* P-element insertion construct (Richter *et al.*, 1996). This final construct was designated *mle-GET*. The sequences of primers used were: 5' primer, 5'-CCTTCCTGTCTCGTAAACCCGCGC-3'; 3' primer, 5'-AATACGGGACAGATGCC-3'; sense mutant primer, 5'-GAAACACAGGGTGCAGAGAGACTACCCAGATTGCC-3'; anti-sense mutant primer, 5'-GGCAATCTGGGTAGTCTCTCCGACCCTGTGTTTC-3'.

Transgenic lines

Plasmid DNA of the *mle-GET* construct was purified by CsCl gradient and injected into *yw*; +; *Ki p^h* [*Js Δ2-3*]/+ embryos (Robertson *et al.*, 1988), according to published protocols (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The G0 progeny were mated to *w*; *pr mle^l*/*CyO* flies. Transgenic male F1 progeny that also contained the *CyO* balancer chromosome were mated to homozygous *pr mle^l* virgin females to determine the chromosome of insertion and the ability of the transgene to rescue homozygous *mle^l* male lethality.

Expression and purification of MLE and *mle-GET*

To express recombinant MLE in Sf9 cells, the coding sequence of *mle25* cDNA (Kuroda *et al.*, 1991) in pBluescript II KS⁺ was subcloned into pET-8c using *NheI* and *BamHI* restriction enzymes. The upstream region of the pET-*mle* plasmid was double-digested with *XbaI* and *NcoI* and replaced by a synthetic linker containing *SpeI* and *NcoI* cloning sites, an initiation codon (ATG) and six continuous CAC codons. This linker was made from two complementary synthetic oligomers. Their sequences were: 5'-TAGAACTAGTACCATTGGGACACCACCACCACCACCA-3' and 5'-TTGATCATGGTACCCTGTGGTGGTGGTGGTGGTGTAC-3'.

The positive recombinant clones, selected by DNA sequencing using the dideoxy chain termination method, were cleaved with *SpeI* and *SmaI* and ligated into the baculovirus vector pBlueBac2. This vector harbored the β-galactosidase gene (Invitrogen) which was treated with *BamHI* and the Klenow fragment followed by *NheI*.

The His-tagged-*mle-GET* recombinant baculovirus was constructed by subcloning a 447 bp *XhoI-SalI* fragment from exon 4 of the *mle-GET* genomic transgene into a pBluescript *mle25* cDNA intermediate. A *HindIII-NorI* fragment of the resulting *mle-GET* cDNA was used to replace the wild-type fragment in a pBlueBacHisB (Invitrogen) construct that contained the *mle25* coding region with the ATG codon engineered as an *NcoI* site (Richter *et al.*, 1996). The final construct was sequenced to verify the presence of the GET mutation. The pBlueBac-*mle-GET* was used to transfect Sf9 cells and the baculoviruses expressing six-histidine tagged recombinant proteins were selected and amplified as described previously (Lee and Hurwitz, 1993).

To produce the recombinant MLE and *mle-GET* proteins, 0.5 l of Sf9 cells (2×10⁶ cells/ml) were infected with the recombinant virus at a multiplicity of 5. Cytoplasmic fractions and nuclear extracts were prepared 48 h after virus inoculation as described previously (Lee and Hurwitz, 1993). Cytoplasmic fractions were adjusted to 0.5 M NaCl and clarified by centrifugation for 30 min at 15 000 r.p.m. in Sorval SS34 rotor. The centrifuged extracts were combined with the nuclear extracts and the mixture was loaded onto a Ni-conjugated Probond column (Invitrogen). All buffers used for the preparation of the recombinant proteins included 50 μM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA), 20 μg/ml aprotinin, 20 μg/ml leupeptin and 10 μg/ml antipain. About 28 ml (245 mg) and 60 ml (507 mg) of crude extracts were obtained from Sf9 cells infected with the recombinant viruses expressing MLE and *mle-GET* proteins respectively. Extracts were loaded onto the Probond column (2 ml) equilibrated with buffer B containing 50 mM Tris-HCl, pH 8.0, 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl and 1 mM phenyl methylsulfonate fluoride (PMSF). After incubation for 1 h at 4°C, the Ni resins were washed with 150 ml of buffer B followed by 150 ml of buffer C (20 mM sodium phosphate, pH 6.0 and 0.5 M NaCl). Bound proteins, including MLE, were eluted with a linear gradient (50 ml) of 0–0.5 M imidazole

in buffer C. For the purification of mle-GET, bound proteins were eluted stepwise by repeatedly applying 1 ml of buffer C containing 0.5 M imidazole. Fractions containing MLE, eluted with 0.25 M imidazole, were pooled (15 ml, 16 mg) and dialyzed overnight at 4°C against 2 l of buffer D containing 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 2 mM dithiothreitol (DTT), 0.1 mM PMSF, 0.05% nonidet P-40 (NP-40) and 10% glycerol. In the case of mle-GET, ~0.12 mg of protein (2 ml) was obtained from the Probond column. Each sample was loaded onto a hydroxylapatite column (3 ml for MLE and 0.5 ml for mle-GET) equilibrated with buffer C. After washing the column with 20 ml of equilibration buffer, a 60 ml of linear gradient (20–700 mM sodium phosphate, pH 7.4, 0.25 M NaCl, 2 mM DTT, 0.05% NP-40 and 10% glycerol) was used for the isolation of MLE and a 10 ml of linear gradient (20–300 mM sodium phosphate, pH 6.0, 0.25 M NaCl, 2 mM DTT, 0.05% NP-40 and 10% glycerol) was used for the isolation of mle-GET. Fractions enriched in recombinant MLE and mle-GET, which were analyzed by SDS–PAGE, were pooled and dialyzed overnight against 2 l of buffer A (20 mM HEPES–NaOH, pH 7.4, 0.25 M NaCl, 0.1 mM EDTA, 0.5 mM PMSF, 4 mM DTT, 12.5% glycerol) and stored in aliquots (0.1 ml) at –70°C. This procedure yields 4.2 mg of MLE (8 ml) and 34 µg of mle-GET (2 ml). Recombinant MLE (53 µg) was loaded onto a 5 ml linear gradient of 20–40% glycerol in buffer A containing 0.5 M NaCl and centrifuged at 45 000 r.p.m. for 36 h at 4°C in a Beckman SW 50.1 rotor. Thirty fractions were collected from the bottom, and aliquots (0.1 µl) were used to assay RNA and DNA helicase activities as described below.

Substrate used for helicase and gel mobility shift assays

Unless otherwise indicated, single-stranded polynucleotide substrates used in the gel mobility shift assay and partial duplex polynucleotides used in the unwinding reaction were prepared as described previously (Lee and Hurwitz, 1992). For the preparation of substrates used to determine the polarity of MLE helicase activity (see Figure 5), four DNA oligomers were synthesized. These included: the 98mer, 5'-GAATACAAGCTTGGGCTGCAGGTCGACTCTAGAGGATCCCCG-GGCGAGCTCGAATTCGGGTCTCCCTATAGTAGTCGTATTAATT-TCGATAAGCCAG-3', corresponding to the longer strand of partial duplex substrates described in Figure 5, A–C; 5'-AGAGTCGACCTGCAGCCCCAAGCTTGTATTC-3', the shorter complementary strand of the 3'-tailed dsDNA substrate, as described in Figure 5B; 5'-CTGGCTTATCGAAATTAATACGACTCATCA-3', the shorter complementary strand of the 5'-tailed dsDNA substrate, as shown in Figure 5C; 5'-GAATACAAGCTTGGGCTGCAGGTCGACTCT-3', the oligomer complementary to the shorter strand of the 3'-tailed dsDNA used for the preparation of the dsDNA substrate described in Figure 5D.

Helicase assays

RNA and DNA helicase activities were measured in reaction mixtures (20 µl) containing 20 mM HEPES–NaOH, pH 7.4, 2 mM DTT, 3 mM MgCl₂, 1 mM ATP, 0.2 mg/ml BSA, 2 units of RNasin (Promega), 50 fmol of dsRNA or dsDNA substrate and the indicated amounts of the helicase fraction. Unless otherwise indicated, the substrate used in the helicase assays was the partial duplex RNA or DNA described in Figure 2. Reaction condition and the quantitation of helicase activities were as described previously (Lee and Hurwitz, 1991). One unit of helicase activity was defined as the amount of protein required to unwind 50 fmol of the partial duplex substrate under the conditions described above.

Gel mobility shift assay

Complexes formed with the recombinant proteins and ssDNA or ssRNA were measured in reaction mixtures (20 µl) containing 20 mM HEPES–NaOH, pH 7.4, 2 mM DTT, 0.2 mg/ml BSA, 50 fmol of each substrate, and the indicated amounts of recombinant protein in the absence or presence of 3 mM MgCl₂ and 1 mM ATP. After 30 min of incubation at 37°C, complexes formed were analyzed and quantitated as described previously (Lee and Hurwitz, 1991).

UV-crosslinking of GTP to MLE and mle-GET

The binding of MLE or mle-GET to GTP was measured in reactions (20 µl) containing 20 mM HEPES–NaOH, pH 7.4, 2 mM DTT, 0.2 mg/ml BSA, 3 mM MgCl₂, 10 µM [α -³²P]GTP (5500 c.p.m./pmol) and the indicated amount of protein. After 10 min of incubation on ice, reaction mixtures in tubes were irradiated for 10 min on ice with a UV-light source (254 nm) 10 cm above the solution. After the addition of 5 µl of SDS sampling buffer, reaction mixtures were electrophoresed through a 7.5% SDS–polyacrylamide gel at 25 mA for 2.5 h. After

overnight fixation in a mixture of methanol and acetic acid (each 10%), the gel was dried on DE-81 paper (Whatman) and the GTP–protein complex was visualized by autoradiography. Following quantitation using the PhosphorImager (FUJIX BAS1000), bands containing GTP were excised from the gel and their radioactivity content was measured by liquid scintillation counting. The radioactivity present in the gel slice containing 80 ng (570 fmol) of MLE was 110 c.p.m., indicating that 20 fmol of GTP was crosslinked to MLE. The extent of GTP-crosslinking to other protein fractions was estimated by comparing their relative intensities measured by PhosphorImager analysis with that obtained with 80 ng of MLE.

Western blot analysis

Aliquots (2 µl) of glycerol fractions containing the recombinant MLE, or protein extracts from whole adult flies and nuclear extracts from Schneider cells, were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose membrane (Palmer *et al.*, 1994). Whole adult fly lysates (approximately one fly/gel lane) and Schneider cell nuclear extracts (~2×10⁵ cells/gel lane) were prepared following the protocol of Palmer *et al.* (1994) and Franke *et al.* (1992) respectively. Affinity purified rabbit anti-MLE12 antibody (Richter *et al.*, 1996) was used at a dilution of 1:2000. The MLE protein was detected using an alkaline phosphatase conjugated goat anti-rabbit IgG detection kit (Promega) for extracts while HRP-conjugated goat anti-rabbit IgG ECL kit (Amersham) was used for the detection of purified proteins.

Polytene chromosome squashes

Polytene chromosome squashes were performed on wild-type Canton S males and *w; pr mle¹*; [*w+; mle-GET*]/+ males. Males heterozygous for the *mle-GET* transgene were generated by crossing: *w; pr mle¹* females to *w; pr mle¹/CyO*; [*w+; mle-GET*] males. Salivary glands were dissected from third instar larvae and the polytene chromosomes were squashed according to standard protocols (Kuroda *et al.*, 1991). The tissue was immunostained according to Pirrotta *et al.* (1988) with a few modifications. Affinity purified rabbit anti-MLE25 antibody (Kuroda *et al.*, 1991) and goat anti-MSL-1 antibody (Palmer *et al.*, 1994) were diluted 1:900 and 1:30 respectively, in PBT (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2% Triton X-100) + 2% BSA and added to the tissue for 16 h at 4°C. The secondary antibodies, FITC or Texas Red conjugated donkey anti-rabbit or anti-goat (Jackson Immuno Research Laboratories Inc.) were diluted 1:200 in PBT + 2% BSA and incubated with the tissue for 4 h at 22°C. After incubation with the secondary antibodies, the tissue was stained with 2 µg/ml bisbenzimidazole (Hoechst 33258, Sigma) for 5 s. The tissue was mounted by placing 2% *n*-propyl galate/80% glycerol between the tissue and a coverslip. The chromosomes were observed using a Zeiss Axioskop and epifluorescence optics and photographed with Ektachrome 400 film.

Acknowledgements

M.I.K. and K.A.C. are grateful for the contributions of R.Richman, S.Fleming, J.Rampersad-Ammons and I.Solovyeva to various phases of the work. We thank R.L.Kelley for critical reading of the manuscript. This research was supported by grants from the N.Y. Heart Association to J.H., and the NIH and Howard Hughes Medical Institute to M.I.K. J.H. is an American Cancer Society Professor. K.A.C. is an HHMI Associate and M.I.K. is an Assistant Investigator of HHMI.

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Received on December 12, 1996; revised on January 23, 1997